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14. ABSTRACT
The overall goal of this project is to determine the underlying mechanisms for the neurological symptoms associated with Gulf War Illness. The central hypothesis is that subthreshold exposures to organophosphates-OPs (defined as exposures not associated with acute signs of toxicity) may have adversely affected axonal transport and/or myelin integrity in affected individuals. We are studying two OPs, a representative insecticide that was used in the first gulf war, chlorpyrifos (CPF), and a representative, nerve agent, diisopropylfluorophosphate (DFP) in rats. The experiments for Specific Aim #1: are now finished. This aim was designed to evaluate OP effects on axonal transport in the living rat brain using manganese-enhanced magnetic resonance imaging (MEMRI) of the optic nerve axonal projections from the retina to the superior colliculus. The following procedures were conducted (N=6): 1) baseline MRI scans; 2) daily injections of vehicle, chlorpyrifos (3.0-18.0 mg/kg) or DFP (0.125-0.5 mg/kg x 14 days); 2) a second MRI scan on the day following the last drug injection; 3) a third scan after a 4 week (OP-free) washout period. For each animal, a separate 6 hour and 24 hour scan was performed after Mn2+ eye injection. For this work one manuscript (the CPF paper) has been published and it is anticipated that a second manuscript (the DFP-MEMRI paper) will be submitted by the end of this year. The experiments for specific aim #2, devoted to the evaluation of OP-related effects on myelin with diffusion tensor imaging-(DTI) and histology are currently underway. A one-year no-cost extension for this project has been granted to allow for the remaining experiments to be finished.

15. SUBJECT TERMS
Organophosphate, Myelin, Axonal Transport, Magnetic Resonance Imaging, Gulf War Illness

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INTRODUCTION

The overall goal of this project is to determine the underlying mechanisms for the neurological symptoms (particularly cognitive deficits) that have been associated with Gulf War Illness. The central hypothesis is that subthreshold exposures to organophosphates (defined as exposures not associated with acute signs of toxicity) from insecticides or nerve agents may have adversely affected axonal transport and/or myelin integrity in affected individuals. We are studying two OPs, a representative insecticide that was used in the first gulf war, chlorpyrifos (CPF), and a representative, nerve agent, diisopropylfluorophosphate (DFP) in rats. The first two years of this proposal were primarily dedicated to Specific Aim #1: which was designed to evaluate OP effects on axonal transport in the living rat brain using manganese-enhanced magnetic resonance imaging (MEMRI) of the optic nerve axonal projections from the retina to the superior colliculus. The last regular year (and now including a one-year no-cost extension to the project) is devoted to diffusion tensor imaging (DTI) and histology experiments for analyzing the effects of repeated OP exposures on myelin.

KEY WORDS

Gulf War Illness, Organophosphate, Pesticide, Nerve Agent, Axonal Transport, Myelin, Manganese Enhanced Magnetic Resonance Imaging, diffusion tensor imaging, learning and memory, cognition

ACCOMPLISHMENTS

Specific Aim 1

The experiments for specific Aim 1 are now completed (chlorpyrifos and DFP exposure-MEMRI studies):

Chlorpyrifos

1. Baseline MRI scans (after Mg^{2+} eye injection) were performed on each test subject.
2. Subjects (N=6) have received daily subcutaneous injections of chlorpyrifos or vehicle (at the following doses) x 14 days:
    - Vehicle
    - CPF 3.0 mg/kg
    - CPF 18.0 mg/kg
3. A second MRI scan was performed on the day following the last drug injection (after another Mg^{2+} eye injection) in each animal.
4. A third scan was performed after a 4 week (OP-free) washout period (after the third and final Mg^{2+} eye injection) in each animal.
5. For each animal a separate 6 hour and 24 hour scan was performed after Mg^{2+} eye injection.
6. We also added an additional set of animals to evaluate the effects of an acute (single) exposure to CPF (18.0 mg/kg N=6) as well a positive control cohort (N=3, intravitreal injection of colchicine).
7. The results of these experiments have been published (see the citation under reportable outcomes below and the manuscript PDF in the Appendix).

DFP

1. Baseline MRI scans (after Mg^{2+} eye injection) were performed on each test subject.
2. Subjects (N=6) have received daily subcutaneous injections of DFP or vehicle (at the following doses) x 14 days:
    - Vehicle
    - DFP 0.125 mg/kg
    - DFP 0.250 mg/kg
    - DFP 0.500 mg/kg
3. A second MRI scan was performed on the day following the last drug injection (after another Mg^{2+} eye injection) in each animal.
4. A third scan was performed after a 4 week (OP-free) washout period (after the third and final Mg^{2+} eye injection) in each animal.
5. For each animal a separate 6 hour and 24 hour scan was performed after Mg^{2+} eye injection.
6. All of the MR scans have been completed and we are still in the process of analyzing these data. We expect to have a publication ready for submission by the end of this year.

<u>Specific Aim 2</u>

The experiments for specific aim #2 are still underway (DTI and histology experiments for analyzing the effects of repeated OP exposure on myelin). The Figure below summarizes the experiments that are ongoing to evaluate the effects of the OPs on myelin. The same OP doses

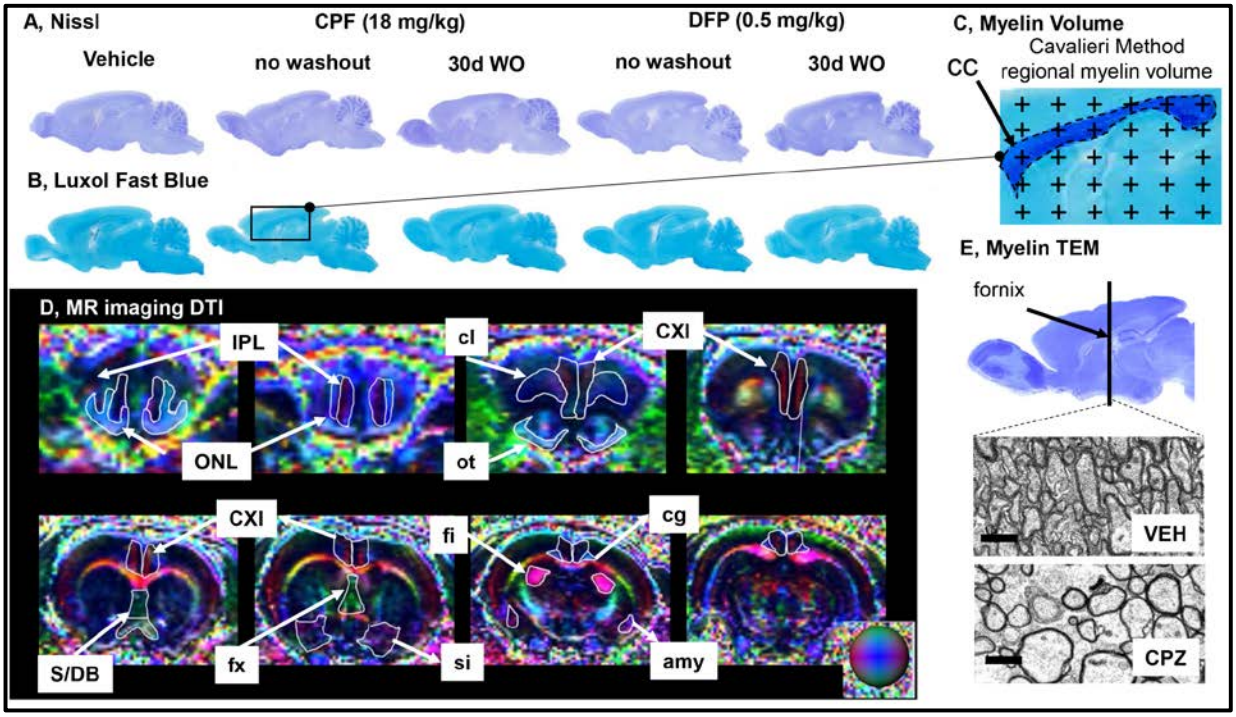

Fig 1. **Summary of ongoing experiments to address Aim 2 of the currently funded project.** Representative images of Nissl (cresyl violet) **(A)** and Luxol fast blue **(B)** stained whole brain sagittal sections from OP treated animals after 14 days exposure. Nissl staining is used to screen for gross morphological changes associated with treatments. **(C)** Unbiased stereological analysis employing the Cavalieri method (point counting) is currently being utilized to measure and compare predominantly myelinated regions such as the corpus callosum (cc) . **(D)** Experiments are in progress to optimize the evaluation of myelination using an MR imaging method, diffusion tensor imaging (DTI) to evaluate forebrain regions associated with learning and memory functions: amygdala (amy), cingulum bundle (cg), claustrum (cl), cerebral cortex lamina I (CXI), fimbria (fi), fornix (fx), inner plexiform layer (IPL), olfactory nerve layer (ONL), ot (olfactory tract), septum/diagonal band (S/DB), substantia innominate (si). The color key to the directionality is indicated at the bottom right. A secondary measure of myelin integrity utilized is transmission electron microscopy (TEM). To date, we have optimized this method using the cuprizone (CPZ) model of global demyelination. **(E)** Representative electron micrographs from the fornix of vehicle and CPZ-treated. scale bars = 1 um

and exposure periods used in Aim 1 are also being evaluated in Aim 2.

Summary of Key Accomplishments:

- As noted above, the experiments for specific Aim 1 have been completed. The chlorpyrifos MEMRI experiments have been published (see citation in the reportable outcomes section below and the manuscript PDF in the appendix). For the DFP MEMRI studies, we are still in the process of analyzing the data, but plan to submit the full publication for review by the end of 2015.
- The data collected to date with both chlorpyrifos and diisopropylfluorophosphate support our hypothesis that repeated exposures to OPs leads to persistent deficits in axonal transport.
- The experiments for specific aim #2 are also underway (DTI and histology experiments for analyzing the effects of repeated OP exposure on myelin).

IMPACT

The diverse array of neurologic symptoms reported by sufferers of GWI suggests that some basic or fundamental neuronal process was adversely affected while these individual were stationed in the Persian Gulf area. The data we have collected in the first 3.0 years of this funded project strongly suggest that repeated exposures to OP-insecticides or nerve agents may have adversely affected axonal transport in these individuals. Specifically, we have now published clear evidence with the insecticide OP, chlorpyrifos to support our hypothesis that repeated OP exposures leads to persistent impairments in axonal transport in the brain of living animals. The data from the DFP study analyzed to date also support this hypothesis. It is expected that the results of these studies will lead to a better understanding of neurobiological basis for the persistent cognitive symptoms of GWI as well as facilitate the development of novel treatments strategies.

CHANGES/PROBLEMS

Some delays have been encountered due to throughput limitations of the Core Imaging Facility for Small Animals (CIFSA), and more recently a defective MRI coil had to be replaced which caused (significant) further delays, particularly in the DTI experiments. Accordingly we have been granted a one year no-cost extension to finish the experiments.

PRODUCTS:

- Publications
    - Hernandez CM, Beck WD Naughton SX, Poddar P, Adam BL, Yanasak N, Middleton C, and Terry AV Jr. Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain. *Neurotoxicology* 47:17-26, 2015.
- Invited presentations
    - "Axonal Transport in Living Rats Exposed to Gulf War Relevant Pesticides". Presentation made at the April meeting of the Research Advisory Committee on

Gulf War Veterans' Illnesses, Veterans Administration (VA) headquarters, 810 Vermont Avenue, Washington, DC. April 20, 2015.

- "Organophosphates and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets". Presented to the Department of Neurobiology and Anatomy, Drexel University, College of Medicine, Philadelphia, PA., June 29, 2015.
- "DFP and Axonal Transport Studies: Overview and Discussion" at the Gulf War Illness Consortium (GWIC) meeting, Boston University School of Public Health, Boston, MA, August 12, 2015.
- "Organophosphate Exposure and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets" Presented at the Tulane University, Neuroscience Program and Department of Pharmacology, New Orleans, LA, September 9, 2015
- "Organophosphate Exposure and Cognitive Deficits: Elucidating the Mechanisms and Identifying Therapeutic Targets" Presented at the University of Georgia, Neuroscience Seminar Series, Athens, GA, October 1, 2015.

- Abstract/Poster Presentations
  - Gao J, Magrane J, Hernandez CM, Terry AV. Exposure to cholinesterase inhibitors leads to persistent impairments of axonal transport in vitro. Abstract/Poster No 97.03/TT38, Washington DC, Society for Neuroscience, November 15, 2014.
  - Hernandez CM, Beck WD, Naughton SX, Poddar I, Adam BL, Yanasak N, Middleton C, Terry AV. Repeated exposures to cholinesterase inhibitors leads to persistent impairments of axonal transport in vivo. Abstract/Poster No. 97.04/TT39, Washington DC, Society for Neuroscience, November 15, 2014.

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Funding Support	

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Funding Support	

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Funding Support	

Other Collaborating Organizations

None

SPECIAL REPORTING REQUIREMENTS

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APPENDICES

Appendix 1 (Published manuscript)

NeuroToxicology 47 (2015) 17–26

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NeuroToxicology

Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain

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ABSTRACT

The toxicity of the class of chemicals known as the organophosphates (OP) is most commonly attributed to the inhibition of the enzyme acetylcholinesterase. However, there is significant evidence that this mechanism may not account for all of the deleterious neurologic and neurobehavioral symptoms of OP exposure, especially those associated with levels that produce no overt signs of acute toxicity. In the study described here we evaluated the effects of the commonly used OP-pesticide, chlorpyrifos (CPF) on axonal transport in the brains of living rats using manganese (Mn^{2+})-enhanced magnetic resonance imaging (MEMRI) of the optic nerve (ON) projections from the retina to the superior colliculus (SC). T1-weighted MEMRI scans were evaluated at 6 and 24 h after intravitreal injection of Mn^{2+}. As a positive control for axonal transport deficits, initial studies were conducted with the tropolone alkaloid colchicine administered by intravitreal injection. In subsequent studies both single and repeated exposures to CPF were evaluated for effects on axonal transport using MEMRI. As expected, intravitreal injection of colchicine (2.5 μg) produced a robust decrease in transport of Mn^{2+} along the optic nerve (ON) and to the superior colliculus (SC) (as indicated by the reduced MEMRI contrast). A single subcutaneous (s.c.) injection of CPF (18.0 mg/kg) was not associated with significant alterations in the transport of Mn^{2+}. Conversely, 14-days of repeated s.c. exposure to CPF (18.0 mg/kg/day) was associated with decreased transport of Mn^{2+} along the ONs and to the SC, an effect that was also present after a 30-day (CPF-free) washout period. These results indicate that repeated exposures to a commonly used pesticide, CPF can result in persistent alterations in axonal transport in the living mammalian brain. Given the fundamental importance of axonal transport to neuronal function, these observations may (at least in part) explain some of the long term neurological deficits that have been observed in humans who have been repeatedly exposed to doses of OPs not associated with acute toxicity.

1. Introduction

The chemicals known as the organophosphates (OPs) are used for a wide variety of important applications and they are especially prevalent in the agricultural setting where they have been applied as pesticides for decades. Unfortunately, OPs are highly toxic to humans as well as target organisms and continuing reports of accidental and intentional poisonings (i.e., from suicide attempts) by OPs is an ongoing environmental and public health concern worldwide (reviewed, Eddleston et al., 2008). The risk of exposure to OP-based nerve agents from rogue governments and terrorist organizations is an additional threat that was recently exemplified by the sarin attacks on civilians in Syria (United Nations Security Council Report, 2013).

The toxic "cholinergic crisis" associated with acute poisoning with OPs and the associated variety of long term neurologic and neurobehavioral consequences have been studied extensively and are primarily attributed to the inhibition of acetylcholinesterase (AChE) (Ecobichon, 2001, for review see also Pereira et al., 2014), However, there is also significant evidence in the human epidemiological literature (e.g., Ross et al., 2013) that OP exposures not associated with acute toxicity may also result in prolonged neurological and neurobehavioral deficits including impairments of cognition. Moreover, as an etiological mechanism, AChE

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0161-813X/

inhibition may not account all of the symptoms associated with acutely toxic or lower level OP exposures as suggested by the following lines of evidence (reviewed in Banks and Lein, 2012): (1) different OPs can have different toxicological profiles despite having similar effects on AChE activity (Bushnell and Moser, 2006; Jett and Lein, 2006; Pope et al., 2005; Pope, 1999), (2) the OP nerve agent, VX induced neurotoxic effects in AChE knockout mice (Duysen et al., 2001); (3) reports in both the human and animal literature indicate that OP toxicity (especially associated with chronic exposure) can occur in the absence of AChE inhibition (Abou-Donia, 2003; Costa, 2006; Kamel and Hoppin, 2004); (4) human studies of occupational exposures to OPs often fail to find a significant correlation between blood cholinesterase activity and neurobehavioral deficits (Rohlman et al., 2011).

Prospective efforts to further elucidate the long term consequences of OP exposures as well as the mechanisms of the deleterious neurological effects require the use of animals and other model systems. Interestingly, more than 30 years ago experiments in animals indicated that axonal transport is negatively affected by OPs, a potentially notable finding given the fundamental importance of axonal transport to neuronal health and brain function. In these early experiments, relatively high doses of phenylphosphonothioate esters and tri-o-cresyl phosphate (i.e., compounds associated with OP-induced delayed neuropathies-OPIDN) inhibited fast anterograde axonal transport in an ex vivo rat optic nerve preparation (Reichart and Abou-Donia, 1980). Later studies in our laboratories indicated that both anterograde and retrograde transport of vesicles in the sciatic nerves (ex vivo) was impaired in rats repeatedly exposed to chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) (CPF), an insecticide OP not associated with OPIDN except at doses well above the LD_{50} (see Richardson, 1995). In these studies doses were used that were not associated with signs of acute toxicity, and further, the axonal transport deficits persisted after a 14-day CPF-free washout period (Terry et al., 2003). Using the same experimental approach, later time course studies indicated that a significant reduction in axonal transport occurred within 10 h of a single CPF exposure (18.0 mg/kg s.c.) (Terry et al., 2007).

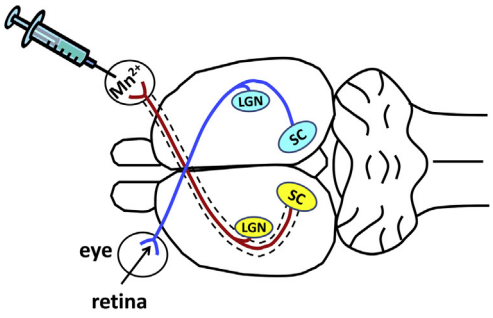
The purpose of the study described here was evaluate the effects of CPF on axonal transport in the brains of living rats using manganese (Mn^{2+})-enhanced magnetic resonance imaging (MEMRI), a non-invasive imaging method that has gained popularity in the last few years. Thus far, the technique has been successfully used to detect impairments of axonal transport in the brains of aged rats (Cross et al., 2008), mouse models of Alzheimer's disease (Kim et al., 2011; Smith et al., 2007, 2011), frontotemporal dementia (Majid et al., 2014), and mice homozygous for a deletion in the amyloid precursor protein gene (Gallagher et al., 2012). MEMRI exploits both the paramagnetic properties of Mn^{2+} and its ability to serve as a calcium analog in neurons. Due to its paramagnetic properties, Mn^{2+} shortens the longitudinal relaxation time constant, T1, of neighboring water, leading to increased intensity in T1-weighted images that can be detected (Lin and Koretsky, 1997) and tracked dynamically over time (Pautler et al., 1998; Pautler and Koretsky, 2002). As a calcium analog, Mn^{2+} enters neurons via voltage-gated calcium channels (Drapeau and Nachshen, 1984; Narita et al., 1990; Sloot and Gramsbergen, 1994; Lu et al., 2007), where it travels within vesicles along microtubules by fast axonal transport (Merritt et al., 1989; Takeda et al., 1998; Silva et al., 2004; Smith et al., 2007; Zhang et al., 2010) in a process that is at least partially dependent on the motor protein kinesin (Bearer et al., 2007, 2009). Here we utilized MRI to visualize Mn^{2+} enhancement of the optic nerve projections (see Lin et al., 2014) from the retina to the superior colliculus. From intravitreal injection, Mn^{2+} has been shown to enter retinal ganglion cells and to travel within their axons in the anterograde direction along the optic nerve to the superior colliculus and lateral geniculate nucleus (Bearer et al., 2007; Watanabe et al., 2001). As a positive control for axonal transport deficits, initial studies were conducted with colchicine administered by intravitreal injection. In subsequent studies, both a single exposure and repeated exposures to CPF administered by subcutaneous injection were evaluated. After the repeated exposure experiments, an extended OP-free washout period was also assessed.

2. Materials and methods

A diagram illustrating the intravitreal injection method and the transport of Mn^{2+} within the axons of retinal ganglion cells in the anterograde direction along the optic nerve to the contralateral superior colliculus and lateral geniculate nucleus is presented in Fig. 1 as well as an overview of the three MEMRI studies described in this report (additional details are provided below).

2.1. Animals

Male albino Wistar rats (Harlan, Indianapolis, IN, USA) 2–3 months old ($n = 6$ per test group) were housed in pairs in a temperature controlled room (25 °C), maintained on a standard 12-h light/dark cycle with free access to food (Teklad Global



Study	Procedure	Imaging	
		6 hrs	24 hrs
1: Colchicine Validation	Mn^{2+} + Colchicine Combined Intravitreal Injection	MRI	MRI
2: CPF Single Expoure	Mn^{2+} Intravitreal Injection CPF s.c. Injection	MRI	MRI
3: CPF Repeated Exposure	Baseline Evaluation Mn^{2+} Intravitreal Injection	MRI	MRI
	↓ CPF s.c. Injection One per day x 14 days		
	↓ 24 hr CPF-Free Washout Mn^{2+} Intravitreal Injection	MRI	MRI
	↓ 30 day CPF-Free Washout Mn^{2+} Intravitreal Injection	MRI	MRI

Fig. 1. Diagram illustrating the intravitreal injection method used in this study and the transport of Mn^{2+} within the axons of retinal ganglion cells (retina) in the anterograde direction (red line) along the optic nerve to the contralateral superior colliculus (SC) and lateral geniculate nucleus (LGN). Below the diagram is the experimental design for each of the three studies described in this report. Abbreviations: chlorpyrifos (CPF), manganese (Mn^{2+}), subcutaneous (s.c.), hours (hrs), magnetic resonance imaging (MRI).

Rodent Diet 2918, Harlan, Madison, WI, USA). All procedures employed during this study were reviewed and approved by the Georgia Regents Sciences University Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

2.2. Drug administration

2.2.1. Colchicine validation study

In order to evaluate our ability to detect axonal transport deficits using MEMRI and our specific experimental conditions, a validation (positive control) study was conducted utilizing colchicine, an agent well-documented to reduce axonal transport in several model systems (see Section 4). Colchicine (Sigma–Aldrich, St. Louis, MO USA) 2.5 μg and $MnCl_2$ 200 μM were co-administered by intravitreal injection in a total volume of 4 μL and MRI scans were collected 6 and 24 h later (for further details, see sections entitled Intravitreal Manganese Administration and Magnetic resonance imaging, below, respectively).

2.2.2. Chlorpyrifos

Chlorpyrifos (CPF) (ChemService, West Chester, PA, USA) was prepared fresh daily in vehicle, a mixture of 3% DMSO (Sigma–Aldrich, St. Louis, MO, USA) and 97% peanut oil (Kroger® Pure Peanut Oil, obtained locally, Augusta, GA, USA) and administered subcutaneously (s.c.) 0.7 ml/kg body weight. The doses evaluated in this study were identified in previous studies (Terry et al., 2003, 2007, 2011; Middlemore-Risher et al., 2010) and operationally defined as doses not associated with acute signs of cholinergic toxicity (e.g., fasciculations, seizures, diarrhea, excessive urination, salivation, etc., see reviews, Rusyniak and Nanagas, 2004; Sungurtekin et al., 2006).

2.2.2.1. Chlorpyrifos single injection study. To evaluate the effects of a single exposure to CPF on axonal transport, subjects were administered CPF (18.0 mg/kg) or vehicle by subcutaneous (s.c.) injection followed immediately with an intravitreal Mn^{2+} (200 μM $MnCl_2$/4 μL) injection. MRI scans were collected 6 and 24 h later.

2.2.2.2. Chlorpyrifos repeated exposure study. To evaluate the effects of repeated exposures to CPF, MEMRI baselines scans were first collected in all test subjects (see below) followed by CPF 3.0 mg/kg, CPF 18.0 mg/kg or vehicle (s.c.) injections once daily for 14 days. MEMRI was subsequently conducted on the day after the last CPF injection (24 h later) then again after a 30-day CPF-free washout period.

In both the single and repeated CPF-exposure experiments described above, individual rats were weighed and monitored (in their home cages for a period of approximately 5 min each day) for visible cholinergic signs (diarrhea, excessive salivation or lacrimation, respiratory difficulties, muscle fasciculations) or other signs of distress throughout the study.

2.3. Intravitreal manganese administration

Rats were anesthetized with a mixture of ketamine, 100 mg/kg i.p. and xylazine, 10 mg/kg i.p. prior to each intravitreal injection. $MnCl_2$ (200 μM/4 μL) was injected with a 30G1½ needle (Beckon-Dickinson Hypodermic #306106) behind the lens to access the vitreous humor of the left eye using care to avoid reflux after removal. Animals were returned to their home cage to be monitored for signs of distress and fully recover prior to each MRI session.

2.4. Magnetic resonance imaging (MRI)

MRI scans were collected 6 and 24 h following each intravitreal Mn^{2+} injection (see Fig. 1A and B). In the CPF repeated exposure study, MRI scans were collected in three separate sessions at the following time points (see Fig. 1B): (1) a pre-treatment baseline, (2) at the end of 14 days of CPF exposure, and (3) following a 30-day CPF-free washout period. Prior to and throughout the MRI imaging session, rats were anesthetized with a mixture of medical air, oxygen (1:1), and 2.5% isoflurane. Once anesthetized, the head was secured (using medical tape) to a thermo-controlled (37.8 °C) cradle while the remainder of the body was unrestrained to promote unrestricted respiration.

Images were acquired on a 7.0 T 20 cm bore BioSpec MRI spectrometer (Bruker Instruments, Billerica, MA, USA). A standard transmit/receive volume coil (35 mm i.d.) was used for imaging. ECG and respiratory signals were monitored by a physiological monitoring system (SA Instruments, INC., Stony Brook, NY). Initial imaging using a three-plane, 2D T1W FLASH sequence (Fast Low Angle Shot: TE/TR = 3.6/145 ms; matrix = 128 × 128; FOV = 3.84 × 3.84 cm^2; flip angle = 30°; five 3.5-mm thick slices per acquisition plane) was performed to prescribe a subsequent high-resolution T1-weighted image as well as to confirm the successful injection of Mn^{2+} into the eyeball. Visualization of the optic nerve enhancement was realized using a 3D FLASH sequence (TE/TR = 3.7/12 ms; # of averages = 10; matrix = 192 × 192 × 192; field of view = 3.84 × 3.84 × 3.84 cm^3; flip angle = 30°). Total scan time was approximately 60 min with total time in the scanner from start to finish being approximately 70 min.

2.4.1. MRI data analysis

2.4.1.1. Mn^{2+} enhancement as a function of distance along the optic nerve. Data analysis software was created in-house using MATLAB (The Mathworks Inc., Natwick, MA, USA). During analysis, the following data were used as input for the software: (1) MRI image data, consisting of 192 × 192 × 192 16-bit raw data files; (2) optic nerve localization masks, constructed as 8-bit binary image files (derived from the MRI image data) pinpointing and isolating the optic nerve from the left eye to the optic chiasm; and (3) cerebellum voxel intensity normalization values, calculated using modifications of methods reported by Minoshima et al. (1993) and Cross et al. (2004). The normalization factors were calculated as follows. First, the cerebellum was outlined to measure the mean, minimum and maximum voxel intensities. Second, the maximum voxel intensity values were sorted to isolate the middle two-thirds for which the mean was calculated. Third, the mean was multiplied by 65% to generate a 'cerebellum voxel normalization value' for each MRI scan. These "normalization procedures were used in order to minimize drift in MR sensitivity and voxel intensity differences across scans obtained at different time points within and between animals.

For each animal at each time point, the analysis software used input data listed above to generate a matching voxel enhancement value at various locations along the optic nerve. First, the optic nerve mask is used to extract optic nerve voxels from the MRI image. Next, a distance algorithm determined the distance between a voxel and the beginning of the optic nerve. Given the distance along the optic nerve for each voxel, voxel intensities were averaged within 0.8 mm bins along the length of the optic nerve. Finally, intensities for all animals were normalized using the ratio of cerebellar intensity to a value of 8000, an estimated mean cerebellum intensity value.

Considering that the optic nerve has a non-zero width, the distance between a voxel and the eyeball was operationally defined as the length along the optic nerve parallel to a curve running through the center of the optic nerve. To begin, the distance algorithm grouped pixel locations within a certain range along the head-to-tail axis in the optic nerve, and calculated a local centroid with coordinates in all three dimensions. A linear interpolation of all local centroids yielded a set of locations that served as the curve along the center of the optic nerve, divided into one thousand sections (i.e., less than the width of a voxel). Given any voxel in the optic nerve map, the closest point along this curve to the voxel was considered to give the distance of the voxel along the optic nerve, where the distance was measured from a coordinate at the beginning of the curve (i.e., the terminus at the eye). A plot of mean voxel intensity as a function of distance along the nerve (originating at the eye) was used to localize the region within which peak enhancement was encountered 6 h after intravitreal Mn^{2+} injection.

2.4.1.2. Mn^{2+} enhancement in the superior colliculus. Each MRI image set was imported as a raw data file (16-bit, $192 \times 192 \times 192$ pixels) and analyzed using a semi-automated method with ImageJ software (Abramoff et al., 2004). Mn^{2+}-enhancement in the superior colliculus (SC) was localized and identified with reference to the Rat Brain Atlas (Paxinos and Watson, 4th edition) to establish neuroanatomical boundaries. Both Mn^{2+} enhanced and contralateral (non-enhanced) SC were manually outlined and analyzed to obtain a mean intensity value, defined as the sum of each voxel's raw intensity value contained with an outline divided by the total number of voxels. For each image, the enhanced SC intensity value was divided by the contralateral non-enhanced SC intensity value to obtain a normalized SC intensity value. In turn, the mean normalized SC intensity was obtained from all image slices in which the SC appeared (10–11 per subject) within each time point for each subject. Data collected from an MRI image set was excluded if one of three events were present, (1) no visible Mn^{2+} enhancement in eyeball or optic nerve in comparison to contralateral side, (2) distorted boundaries of SC or optic nerve due to movement (i.e., motion artifact), and (3) no Mn^{2+} enhancement in SC during baseline scan (repeated exposure CPF group only). In any case where an MRI image set was excluded due to the criteria described above, the particular subject was removed from the study and replaced with a new animal (i.e., the entire series of experiments was repeated). For each experimental group evaluated in the current study 2–3 animals were replaced.

2.5. Acetylcholinesterase activity

Acetylcholinesterase activity was assessed in brain using the method of Ellman (Ellman et al., 1961) with modifications to accommodate a 96-well microplate format at 25 °C (Terry et al., 2007). Brains were collected in separate sets of animals in parallel with the animals in the MEMRI studies at four time points, (1) 6 and (2) 24 h following a single CPF (18.0 mg/kg, s.c.) injection, (3) 24 h after completing 14 days of repeated CPF exposure (3.0 or 18.0 mg/kg, s.c.), and (4) 30 days after completing 14 days of repeated CPF exposure (3.0 or 18.0 mg/kg, s.c.). Subjects were anesthetized with isoflurane and transcardially perfused with ice-cold phosphate-buffered saline (PBS) to thoroughly clear the brain of blood, particularly peripheral borne butyrylcholinesterases. Brains were extracted, rinsed with PBS and stored at −80 °C until use. Brain tissue was homogenized in ice-cold PBS (wt/vol: 1 g wet brain tissue/4 mL PBS) using a motor driven glass-teflon tissue grinder and total protein concentration was measured using a Micro BCA Protein Assay Kit (ThermoFisher Scientific Inc., Rockford, IL, USA) according to manufacturer's instructions. Brain homogenates (20–50 μg protein/μl) were assayed in triplicate for AChE using 0.48 mM acetylthiocholine (substrate) and 0.52 mM dithiobisnitrobenzoic acid diluted in 1.0 mM sodium phosphate buffer (see Terry et al., 2007). The formation of reaction product (yellow color) was monitored by measuring absorbance values (in optical density) at 412 nm every 2 min for 16 min (μQuant™ Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). The cholinesterase-mediated reaction rate (moles/L per min) was calculated by dividing the change in absorbance per min by 13,600 (Ellman et al., 1961).

2.6. Statistical analyses

All statistical analyses were performed using NCSS 2001 (NCSS, Kayesville, UT, USA). Mn^{2+}-enhancement, body weights and cholinesterase activity were compared by Student's *t*-tests or analysis of variance with repeated measures when indicated to compare treatment (vehicle vs. colchicine or chlorpyrifos), time (baseline, washout 1 or washout 2) and/or distance along optic nerve (in 0.8 mm bins). Student–Newman–Keuls multiple comparison procedures were used to examine post hoc differences when indicated. Statistical significance was assessed using an alpha level of 0.05.

3. Results

3.1. Body weights

Test subjects were weighed upon arrival, then again on the day of dosing in the colchicine and single exposure CPF study. The weights of the animals in these studies ranged from approximately 325–370 g at the time of dosing and were not significantly different. In the repeated exposure study, the subjects were weighed at different time points (i.e., at baseline, the end of CPF dosing, and at the end of the 30-day CPF-free washout period, see Table 1A). Both vehicle and CPF-treated subjects gained weight as expected over the course of the repeated exposure study (effect of weighing day, $F_{13,295} = 92.83$, $p < 0.001$). Please note that the statistical values provided in the parentheses in the preceding sentence and below refer to the *F* statistic followed by the between-groups degrees of freedom and the within-groups degrees of freedom, respectively. CPF (18.0 mg/kg)-treated subjects had gained slightly (albeit not significantly) less weight than the other two experimental groups after 14 days of treatment, but all groups were similar after the 30-day CPF-free washout period.

3.2. Signs of distress or cholinergic toxicity

As noted in the Methods, in both the single and repeated CPF-exposure experiments, individual test subjects were weighed and monitored daily for visible cholinergic symptoms and/or other signs of distress throughout the study. There were no cases where such signs were detected in any portion of the study.

3.3. Acetylcholinesterase (AChE) activity

The effects of single and repeated exposure to CPF on AChE activity are provided in Table 1B. A single exposure to CPF was not associated with significant inhibition of AChE activity at 6 or 24 h post injection. In the CPF (14-day) repeated exposure study, AChE activity was reduced (24 h after the last injection) in CPF-treated rats ($F_{2,12} = 159.30$, $p < 0.001$) to approximately 60% and 20% of control in the 3.0 and 18.0 mg/kg group, respectively (post hoc $p < 0.05$ for both doses). After the 30-day CPF-free washout period, AChE activity had fully returned to control levels in the lower dose

Table 1
Body weights and acetylcholinesterase activity.

A. Body weights

Group	Repeated exposure study				
	Baseline (BL)	Washout 1	% Change from BL	Washout 2	% Change from BL
Vehicle	328.89 ± 7.16	392.00 ± 5.74	14.33 ± 1.42	481.00 ± 9.43	45.89 ± 2.43
CPF 3.0 mg/kg	331.00 ± 7.55	382.50 ± 6.52	15.71 ± 2.33	479.33 ± 9.82	39.33 ± 1.02
CPF 18.0 mg/kg	348.38 ± 12.31	366.57 ± 13.30	8.67 ± 3.57	462.29 ± 16.52	41.75 ± 3.97

B. Acetylcholinesterase activity

Group	Acute exposure study			
	6 h time point	% of control	24 h time point	% of control
Vehicle	37.59 ± 1.80		33.10 ± 4.88	
CPF 18.0 mg/kg	40.57 ± 1.52	107.93 ± 4.04	33.94 ± 4.06	104.20 ± 8.83

Group	Repeated exposure study			
	Washout 1	% of control	Washout 2	% of control
Vehicle	37.38 ± 1.27		34.35 ± 0.86	
CPF 3.0 mg/kg	22.56 ± 1.52**	60.36 ± 4.01	34.85 ± 0.83	101.46 ± 2.42
CPF 18.0 mg/kg	7.32 ± 0.58**	19.59 ± 1.56	25.44 ± 0.71**	74.06 ± 2.06

Acetylcholinesterase activity is expressed as nmoles acetylthiocholine hydrolyzed/min/mg protein.
Abbreviations = CPF, Chlorpyrifos.
** $p < 0.001$ versus vehicle at the time point indicated.

group, whereas it was still reduced to approximately 74% of control in the higher dose group ($F_{2,12} = 43.46$, $p < 0.001$, post hoc $p < 0.05$).

3.4. Colchicine study

The effects of colchicine (or vehicle) administered by intravitreal injection on axonal transport are illustrated in Fig. 2. Both experimental groups (Fig. 2A) demonstrated decreases in signal intensity in the optic nerves the further away from the eyeball, main effect of distance, ($F_{13,76} = 8.25$, $p < 0.001$) 6 h after intravitreal Mn^{2+} injection. However, colchicine-treated rats exhibited significantly diminished Mn^{2+} enhancement along the optic nerve from the eyeball to the optic chiasm compared to vehicle-treated rats, main effect of group, ($F_{1,76} = 9.00$, $p < 0.05$). Post hoc analysis indicated that enhancement was significantly lower ($p < 0.05$) at all points past the initial 0.8 mm segment in the colchicine-treated subjects compared to vehicle-treated controls. This effect on the Mn^{2+}transport is also clearly apparent 24 h later in the superior colliculus (Fig. 2B), where enhancement is visibly reduced in colchicine-treated rats compared to vehicle-treated controls ($p < 0.001$).

3.5. Chlorpyrifos single exposure study

The effects of a single (subcutaneous) injection of CPF (18.0 mg/kg), compared to vehicle-treated controls on axonal transport are illustrated in Fig. 3. Six hours after intravitreal injection of Mn^{2+} both experimental groups demonstrated progressive decreases in signal intensity in the optic nerves the further away from the eyeball, main effect of distance ($F_{13,167} = 3.48$, $p < 0.001$), particularly the latter 5.6 mm most proximate to the optic chiasm (Fig. 3A). However, there were no statistically significant effects of CPF on the transport of Mn^{2+} (main effect of group $p > 0.05$). In addition, there were also no differences in signal intensity observed in the superior colliculus 24 h after intravitreal injection of Mn^{2+} (Fig. 3B).

3.6. Chlorpyrifos repeated exposure study

The effects of repeated exposure to CPF (3.0 mg/kg, 18.0 mg/kg, or vehicle administered by subcutaneous injection once daily for 14 days) on axonal transport are illustrated in Figs. 4 and 5. In each of the figures the following conditions are illustrated: baseline (BL)-before CPF or vehicle exposure, washout 1 (WO1)-one day after the last drug injection; washout 2 (WO2)-30 days after the last drug injection. For each condition, Mn^{2+} contrast (6 h after intravitreal injection) along the optic nerve (Fig. 4) was greatest within the initial 4.0 mm from the eyeball and then significantly decreased closer to the optic chiasm, main effect of distance, ($F_{13,949} = 57.07$, $p < 0.001$). The following main effects were also observed for the optic nerve comparisons, group x time x distance, $F_{52,949} = 1.91$, $p < 0.001$. Post hoc analyses indicated that in the subjects treated with CPF 3.0 mg/kg, enhancement was significantly decreased (compared to their respective baseline values, $p < 0.05$) at washout 1 in the initial four, 0.8 mm bins from the eyeball (i.e., at 0.8, 1.6, 2.4, and 3.2 mm). At washout, 2 significant (or nearly significant) decreases were observed in the CPF 3.0 mg/kg treated subjects at the 1.6 and 3.2 mm distances from the eyeball ($p = 0.002$ and 0.07, respectively). While a trend toward reduced signal intensity can also be observed in the subjects treated with CPF 18.0 mg/kg between the 2 and 4 mm distances from the eyeball at washout 2, the differences did not meet the required level of significance (p values ranged from 0.06 to 0.10). In the superior colliculus (24 h after intravitreal Mn^{2+} injection, Fig. 5), the following main effects were observed, group effect, $F_{2,60} = 4.012$, $p < 0.05$. Post hoc analysis indicated that signal intensity was lower ($p < 0.05$) at WO1 for both the 3.0 and 18.0 mg/kg CPF groups compared to vehicle-treated controls. In addition, within group comparisons indicated that CPF 18.0 mg/kg, but not 3.0 mg/kg was associated with reduced Mn^{2+} enhancement ($p < 0.05$) in the superior colliculus at both WO1 and WO2 compared to the respective baseline values.

4. Discussion

As noted in the Introduction, the main purpose of this study was to determine if repeated exposures to a commonly used insecticide OP (CPF) were associated with impairments in axonal transport in the brain of living rats as we have previously observed in peripheral (ex vivo) nerve preparations (Terry et al., 2003, 2007). The initial experiments with colchicine confirmed our ability to use MEMRI as a suitable method for detecting axonal transport deficits in the brains of rats. Colchicine is a tropolone

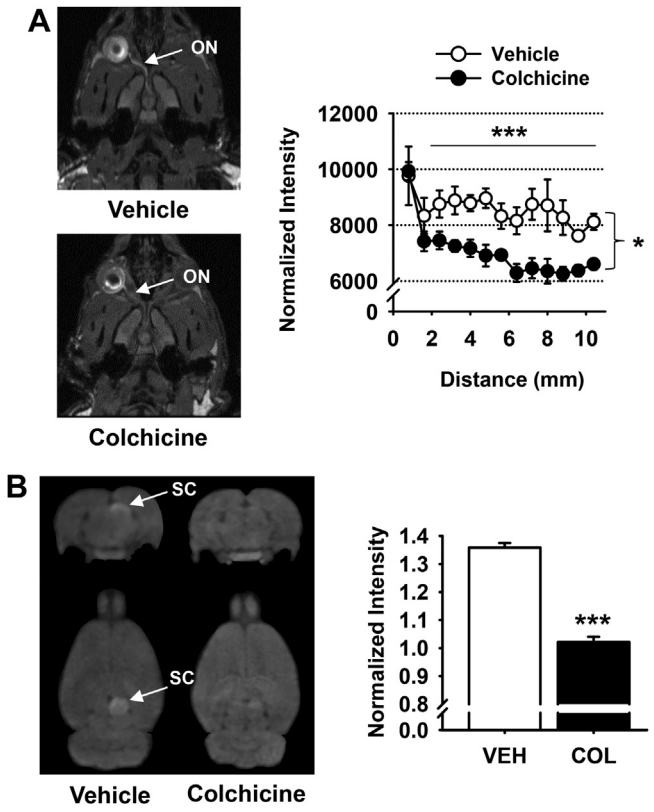


Fig. 2. Colchicine validation study. Colchicine 2.5 μg and $MnCl_2$ 200 μM were co-administered by intravitreal injection and MRI scans were collected 6 and 24 h later. (A) Representative horizontal (axial) T1-weighted MR images of rat brain (proximate to interaural line) of the optic nerve emerging from the eyeball (6 h). Note the clear Mn^{2+} enhancement along the full length of the right optic nerve (indicated by white arrows) to the optic chiasm of the vehicle but not in the colchicine-treated subject. The line graphs to the right illustrate normalized intensity values (see methods) in 0.8 mm bins along the optic nerve from the eye to the optic chiasm. (B) Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma −3.86 mm) MR images. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) of vehicle but not in the colchicine-treated subject. A histogram illustrating the normalized intensity values (mean ± s.e.m.) is provided in the right part of the figure. $^{*}p < 0.05$, colchicine vs vehicle, $^{***}p < 0.001$ initial 0.8 mm vs remaining 10.4 mm (in 0.8 mm bins) of optic nerve. Abbreviations: colchicine (COL), hour (h), magnetic resonance imaging (MRI), manganese (Mn^{2+}), optic nerve (ON), superior colliculus (SC), vehicle (VEH) ($N = 3$).

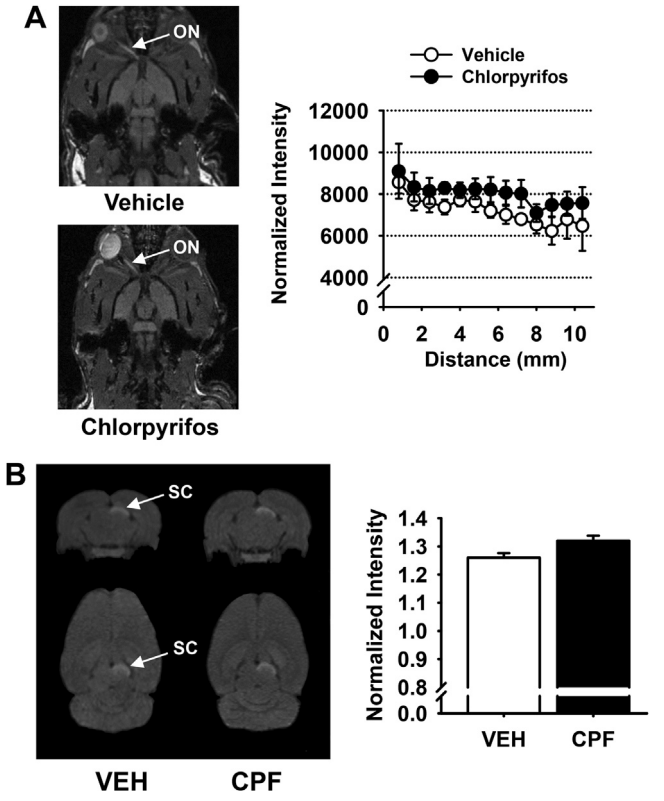


Fig. 3. Chlorpyrifos (CPF) Acute Study. Intravitreal Mn^{2+} injections were immediately followed by a single dose of CPF (s.c., 18.0 mg/kg), then MRI scans were collected 6 and 24 h later. **A.** Representative horizontal (axial) T1-weighted MR images of rat brain (proximate to interaural line) of the optic nerve emerging from the eyeball (6 h). Note the clear Mn^{2+} enhancement along the full length of the left optic nerve (indicated by white arrows) to the optic chiasm of the vehicle and CPF-treated subjects. The line graphs to the right illustrate normalized intensity values (see methods) in 0.8 mm bins along the optic nerves to the optic chiasm. (B) Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma −3.86 mm) MR images. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) 24 h following injection of the vehicle and CPF-treated subjects. A histogram illustrating the normalized intensity values (mean ± s.e.m.) is provided in the right part of the figure ($N = 6$).

alkaloid that binds tightly to tubulin thus impairing tubulin polymerization and the assembly of microtubules. The consequent disruption of microtubule dynamics impairs the ability of motor proteins to transport cargo in axons (Hastie, 1991; Uppuluri et al., 1993; Han et al., 1998). The intravitreal administration of colchicine at a dose of 2.5 μg was chosen for use in our experiments based on the work of Karlsson et al. (1971), as it was the lowest dose associated with impaired axonal transport in the retinal ganglion cells of rabbits. In each of the 3 studies described in this report, MRI scans were collected 6 and 24 h following each intravitreal Mn^{2+} injection so that quantitative comparisons of transport could be made in the optic nerve and superior colliculus. This was based on previous studies where, using intravitreal Mn^{2+} injections and MEMRI of the rat visual pathway, the rate of Mn^{2+} transport within retinal ganglion cell axons was estimated at approximately 1 mm/hr and the entire visual projection from the retina to the superior colliculus was enhanced within 24 h (Thuen et al., 2005, 2008).

The next series of experiments were conducted to determine if a single exposure to CPF affected axonal transport of Mn^{2+} in the visual pathway. This was based on a previous series of experiments where we observed a significant reduction in anterograde and retrograde axonal transport of vesicles in the sciatic nerves (ex vivo) of rats within 10 h of a single (s.c.) injection of CPF 18.0 mg/kg (Terry et al., 2007). Surprisingly, we did not observe any statistically significant alterations of Mn^{2+} transport in the optic nerve at 6 or 24 h after intravitreal injection of Mn^{2+} nor did we observe significant differences in Mn^{2+} enhancement in the superior colliculus at the 24 h time point. The basis for this unexpected observation is unclear, but could be related to some anatomical differences between retinal ganglion cells and neurons in the sciatic nerve or the easier (or more rapid) access to neurons by CPF in the periphery versus the CNS.

Conversely, in the 14-day repeated exposure experiments, Mn^{2+} transport to the superior colliculus was clearly decreased by CPF (most notably at the 18.0 mg/kg dose), an effect that was also present after a 30-day (CPF-free) washout period. There was also evidence of CPF-related transport deficits in the optic nerves, specifically; the 3.0 mg/kg (but not the 18.0 mg/kg) dose of CPF was associated with statistically significant decreases in transport of Mn^{2+}. The source of the dose/washout time discrepancy in the optic nerves is unclear, although from visual inspection of Fig. 4 it does appear that signal intensity is reduced (albeit non-significantly) at multiple points in washout 2 in the CPF

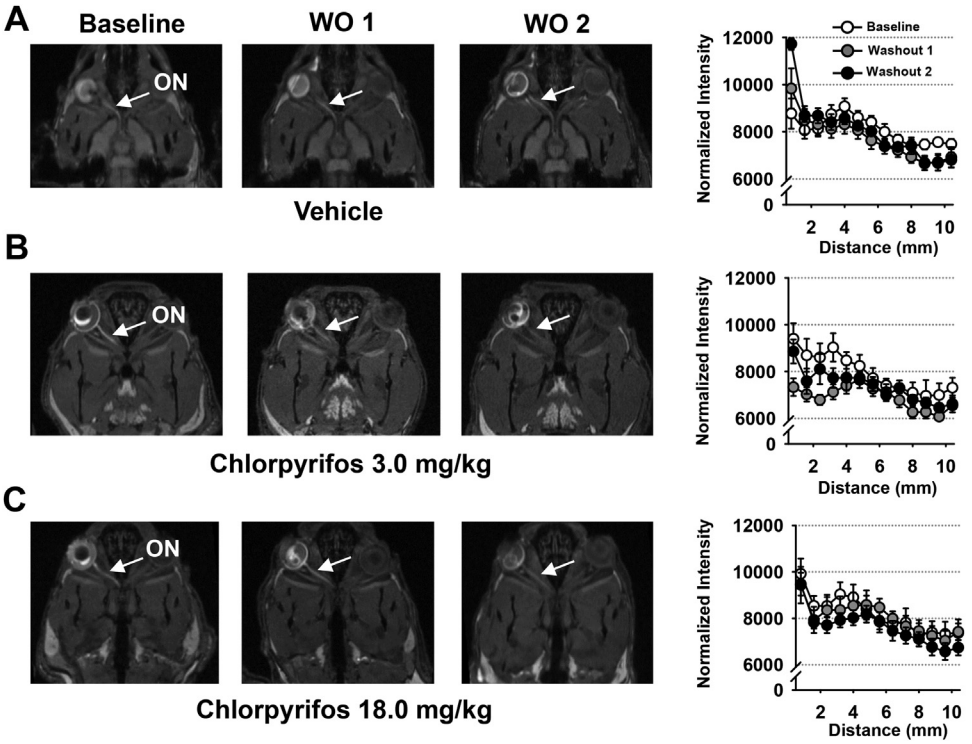


Fig. 4. Chlorpyrifos (CPF) Repeated Exposure Study-Optic Nerve Images. Baseline MRI scans were obtained for each test subjects and then CPF (3.0 and 18.0 mg/kg), or vehicle was administered by subcutaneous injection once daily for 14 days. One day (24 h) after the last CPF injection $MnCl_2$ was administered by intravitreal injection and MRI scans were collected 6 h later. After a 30-day CPF-free washout period, the MEMRI procedure was repeated. In the figure, representative horizontal (axial) images illustrating the Mn^{2+} contrast in the optic nerves (ON, indicated by white arrows) are provided. From left to right, the following conditions are illustrated: baseline (BL), before CPF or vehicle exposure, washout 1 (WO1)-one day after the last CPF injection; washout 2 (WO2)-30 days after the last CPF injection and a corresponding line graph illustrating normalized intensity values (see methods) in 0.8 mm bins along the optic nerves from the eyeball to the optic chiasm for each treatment group: (A) vehicle, (B) CPF 3.0 mg/kg and (C) CPF 18.0 mg/kg. Note the reduced signal intensity within the first 4 mm of the optic nerves in the line graphs in the CPF 3.0 mg/kg-treated subjects at WO1 and WO2 compared to their respective baseline values ($N = 6$).

(18.0 mg/kg)-treated subjects. There was one other observation in this study that may suggest that the measurements in the superior colliculus 24 h after Mn^{2+} injection are more reliable for optimal comparisons of axonal transport than the optic nerve comparisons at 6 h. Specifically, under vehicle control conditions in the colchicine study (Fig. 2) the normalized intensity values decreased at 2 mm and beyond from the eyeball, but plateaued around 8000, while the vehicle-treated animals in both CPF studies (Figs. 3 and 4) showed greater decreases in intensity as one moved further away from the eyeball. It should be noted that in the colchicine study only 3 animals per group were analyzed and it may simply be that transport in these animals was slightly higher than what is more commonly observed. While, as noted above, we based the 6 h optic nerve measurements on a Mn^{2+} transport rate of 1 mm/hr (from previous reports), the 6 hr time point may have been too long for optimal quantitative comparisons. Overall, however, these results clearly indicated that colchicine served well as a positive control for producing axonal transport deficits and that repeated exposures to CPF also lead to persistent deficits in transport. These later results (in the living rodent brain) would appear to support the aforementioned studies (Terry et al., 2003, 2007) where repeated exposures to doses of CPF well below those associated with acute toxicity led to persistent impairments in the transport of vesicles in the sciatic nerves of rats.

The basis for the persistence of the effects of CPF on axonal transport observed in this study (and the previous sciatic nerve studies) is unclear. While most insecticide OPs are lipophilic (Vale, 1998) and prone to sequester in fatty tissues such as the brain, it has been argued that chlorine-containing OPs such as CPF have further enhanced lipid solubility leading to delays in elimination from the body (Blodgett, 2006). In one of our previous studies (Terry et al., 2007), CPF was easily detectible in the rat brain (mean ~5 nmoles/g) up to 14 days after the last (18.0 mg/kg) injection in a repeated exposure regimen. Further, in the present study AChE continued to be inhibited (~26%) at 30 days after the last exposure to the 18.0 mg/kg dose of CPF, again supporting the argument the CPF persists for long periods in vivo. Importantly, while we have used the term "washout" for simplicity in this study to reflect the periods where no CPF was injected, this should not be interpreted as if there was no CPF remaining in the brain in vivo. While we have observed deficits of axonal transport in vitro with additional OPs (e.g., the nerve agent, DFP, personal communication) it is unclear if this type of persistent effect on axonal transport (in vivo) would occur with other OPs of different structural classes and levels of lipid solubility.

While this study was not designed to investigate potential molecular mechanisms for OP-related alterations in axonal transport, one hypothesis is that OPs might (in some manner) alter the function of motor proteins such as kinesin and dynein and/or components of the neuronal cytoskeleton (e.g., microtubules) that are important for axonal transport (reviewed, Terry, 2012). As noted in the Introduction, previous studies have indicated that the transport of Mn^{2+} in vesicles along microtubules is at least partially dependent on the motor protein kinesin (Sloot and Gramsbergen, 1994; Takeda et al., 1998). The hypothesis that OPs might interact with kinesin and negatively affect kinesin-driven axonal transport is supported by previous in vitro studies (microtubule motility assays) in our laboratories (Gearhart et al., 2007) as well a mass spectrometry studies in other laboratories where (using the biotin-tagged OP agent, FP-biotin) OP binding to tyrosine in the human kinesin 3C motor domain was demonstrated (Grigoryan et al., 2009). An alternative (or perhaps complementary) hypothesis

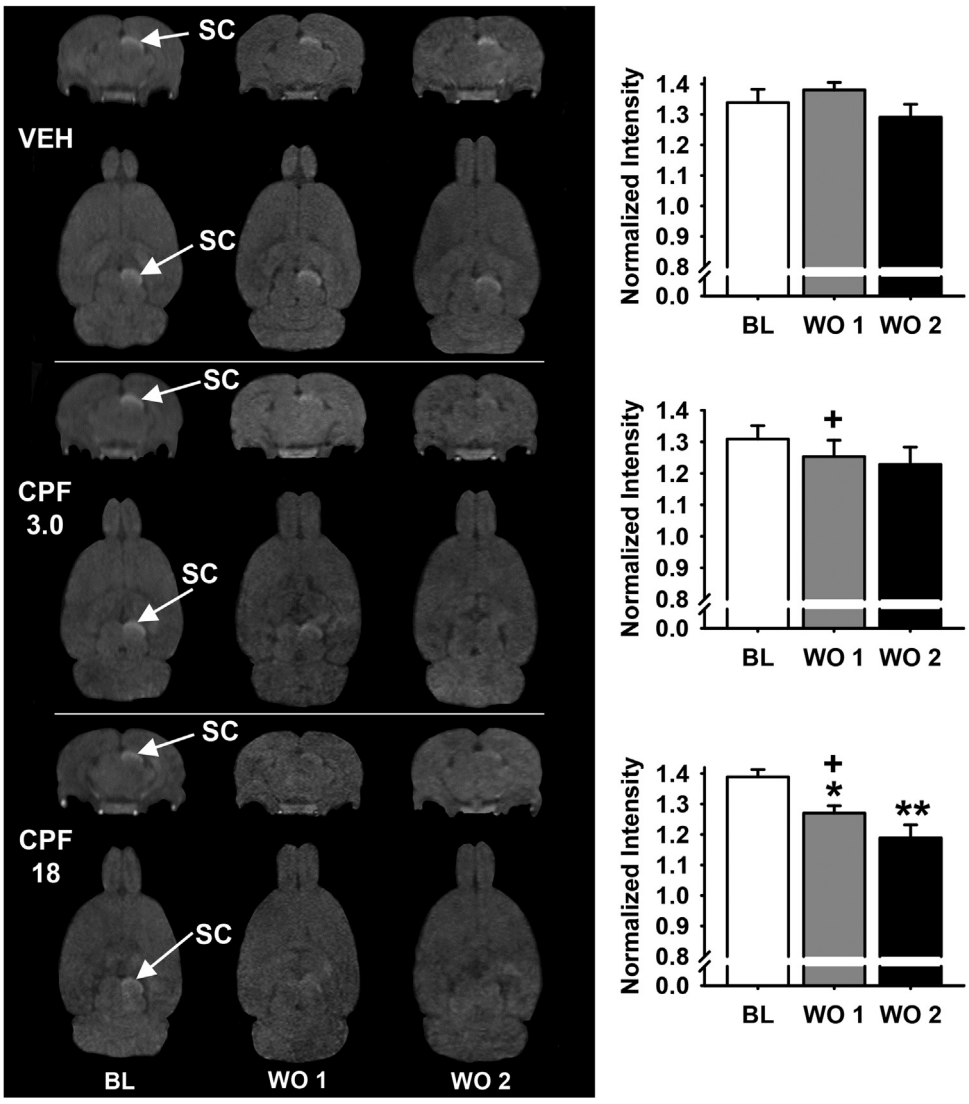


Fig. 5. Chlorpyrifos (CPF) Repeated Exposure Study-Brain Images. Baseline MR images were obtained from test subjects and then CPF (3.0 and 18.0 mg/kg), or vehicle was administered by subcutaneous injection once daily for 14 days. One day (24 h) after the last CPF injection and 30-days after a CPF-free washout period, MRI scans were collected 24 h after intravitreal Mn^{2+} injection. Representative rat brain coronal (Top, approximately interaural 2.70 mm, bregma 6.30 mm) and horizontal (Bottom, approximately interaural 6.14 mm, bregma −3.86 mm) MR images from each group and time point. From left to right, the following conditions are illustrated: baseline (BL), before CPF or vehicle exposure, washout 1 (WO1)-one day after the last CPF injection; washout 2 (WO2)-30 days after the last CPF injection. Note the clear Mn^{2+} enhancement in the superior colliculus (indicated by white arrows) of all groups at baseline and reduced enhancement associated with CPF (18.0 mg/kg) at WO1 and WO2. Histograms illustrating the normalized intensity values (mean ± s.e.m.) are provided to right of each MRI. $^{*}p < 0.05$; $^{**}p < 0.01$ CPF-WO1 and -WO2 versus baseline values, respectively. $^{+}p < 0.05$ versus vehicle control at the same washout period ($N = 6$).

is that (like colchicine) OPs impair tubulin polymerization leading to the disruption of microtubule assembly which in turn leads to impairments of axonal transport. This hypothesis is supported previous spectrophotometric studies (Prendergast et al., 2007) and experiments using atomic force microscopy (Grigoryan and Lockridge, 2009) where CPF-oxon was found to disrupt tubulin polymerization. Interestingly CPF-oxon has also been shown to covalently bind to tubulin, an effect that may explain the disruptions in tubulin polymerization (Jiang et al., 2010).

The observations in animals and in vitro models described above suggest that OP-related effects on axonal transport represent one attractive hypothesis for the wide variety of long-term neurological symptoms that have been associated with OP exposure in humans. Axonal transport is an essential process in neurons that is responsible for the movement of a variety of important macromolecules (e.g., mitochondria, receptor proteins, growth factors) to and from a neuron's cell body (reviewed, Duncan and Goldstein, 2006) and further, impairments in axonal transport have been implicated in the pathology of a wide variety of neurological illnesses (e.g., amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, Parkinson's disease, Pick's disease, and progressive supranuclear palsy, see Stokin and Goldstein, 2006 for review). It is noteworthy that many of these illnesses are characterized by similar neurobehavioral deficits that have been observed in people who have been exposure to OP-based pesticides. As an example, a recent meta-analysis of 14 studies and data from more than 1600 participants, found an association between OP exposures (below those associated with acute toxicity) and impairments in attention, working memory, executive function, visuospatial ability, and visual memory (Ross et al., 2013). Here it is also important to note that there is a small but growing body of literature to suggest that OP exposure may even represent a potential risk factor for Alzheimer's disease as well as some of the other neurodegenerative disorders mentioned above (Hayden et al., 2010; Hancock et al., 2008; Zaganas et al., 2013).

A similar constellation of chronic neurologic symptoms to that discussed in the preceding paragraph has also been associated with the syndrome now known as Gulf War Illness (GWI, Lange et al., 2001) which has been consistently observed in about 25–30% of Gulf War veterans, or about 175,000–250,000 of the 700,000 troops deployed to the war in 1990–1991. Interestingly, among the potential contributing factors to GWI, exposures to OP-based insecticides and nerve agent-OPs (following the destruction of an Iraqi munitions storage complex at Khamisiyah, Iraq, in March 199) have been implicated (RAC, 2014).

It is also important to note that several prospective behavioral studies in our laboratories and others have found OP-related deficits in behavioral tasks that map well onto the domains of cognition that have been found to be affected in humans with GWI and/or those who are known to have been previously exposed to OPs. These animal tasks include delayed matching (working memory), water maze (spatial learning and memory) novel object recognition (recognition memory), and the performance of the five-choice serial reaction time task (sustained attention) (Bushnell et al., 1991; Terry et al., 2003, 2007, 2011; Middlemore-Risher et al., 2010; Yan et al., 2012; Terry et al., 2014).

In conclusion, the results of this animal study indicate that repeated exposures to a commonly used OP pesticide, CPF can result in persistent alterations in axonal transport in the living mammalian brain. Given the fundamental importance of axonal transport to neuronal function, these observations may (at least in part) explain some of the long term neurological deficits that have been observed in humans who have been repeatedly exposed to OPs. These findings may complement other studies which have identified other deleterious effects of OPs that may be additive (or unrelated) to AChE inhibition and include oxidative stress, impairments of mitochondrial function, neuroinflammation, altered neurotrophin responses, etc. (reviewed, Soltaninejad and Abdollahi, 2009; Banks and Lein, 2012; Terry, 2012).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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